NORGEN BIOTEK CORPORATION

3430 Schmon Parkway
Thorold, ON, Canada L2V 4Y6
Phone: 866-667-4362 • (905) 227-8848
Fax: (905) 227-1061
Email: techsupport@norgenbiotek.com

Mycoplasma PCR Detection Kit — Product Insert

Product # 33100

Pathogen Information

Mycoplasma is a common and serious contaminants of cell cultures. *Mycoplasma* is often not visible and does not respond to antibiotics, and therefore it is a major issue that requires monitoring and early detection. Up to 30% of cell cultures may be contaminated with *Mycoplasma*s, the main contaminants being the species *M. orale*, *A. laidlawii*, *M. arginini* and *M. hyorhinis*. These organisms produce a variety of effects on the infected cells in culture, including changes in metabolism growth, viability and morphology, thereby altering the phenotypic characteristics of the host cells. Therefore there is an absolute requirement for routine, periodic assays to diagnose possible *Mycoplasma* contamination of all cell cultures, particularly continuous or established cell lines.

Principle of the Test

Norgen's *Mycoplasma*PCR Detection Kit constituents a ready-to-use system for the isolation and detection of *Mycoplasma* using end-point PCR. The kit first allows for the isolation of *Mycoplasma* DNA from the blood samples using spin-column chromatography. The *Mycoplasma* DNA is isolated free from inhibitors, and can then be used as the template in a PCR reaction for *Mycoplasma* detection using the provided *Mycoplasma* Master Mix. The *Mycoplasma* Mastermix contains reagents and enzymes for the specific amplification of a 327 region of the genome. In addition, Norgen's *Mycoplasma* PCR Detection Kit contains a second Mastermix, the PCR Control Master Mix, which can be used to identify possible PCR inhibition and/or inadequate isolation via a separate PCR reaction with the use of the provided *PCR control (PCRC)* or *Isolation Control (IsoC),* respectively. This kit is designed to allow for the testing of 24 samples.

Kit Components:

Component	Contents
Lysis Solution	15 mL
Wash Solution	9 mL
Elution Buffer	3 mL
Bead Tubes	24
Mini Spin Columns	24
Collection Tubes	24
Elution tubes (1.7 mL)	24
Mycoplasma 2x Detection PCR Master Mix	0.35 mL
Control 2x PCR Master Mix	0.35 mL
Isolation Control (IsoC)[*a]	0.3 mL
Mycoplasma Positive Control (PosC)[*b]	0.1 mL
Nuclease Free-Water	1.25 mL
Norgen's DNA Marker	0.1 mL
Product Insert	1

* *IsoC = Isolation Control ; PosC= Positive Control*
[a] *The isolation control is a cloned PCR product.*
[b] *The positive control is a fragment of Mycoplasma cloned in a plasmid*

Customer-Supplied Reagents and Equipment

- Disposable powder-free gloves
- Benchtop microcentrifuge
- 1.5 mL microcentrifuge tubes
- 65°C water bath or heating block
- 96 – 100% ethanol
- 70% ethanol

Storage Conditions and Product Stability

All solutions should be kept tightly sealed and stored at room temperature. These reagents should remain stable for at least 1 year in their unopened containers.

The Mycoplasma 2x PCR Master Mix, Control 2x PCR Master Mix, Mycoplasma Positive Control (*PosC*) and the Isolation Control (*IsoC*) should be kept tightly sealed and stored at -20°C for up to 1 year without showing any reduction in performance. Repeated thawing and freezing (> 2 x) should be avoided, as this may reduce the sensitivity. If the reagents are to be used only intermittently, they should be frozen in aliquots.

General Precautions

The user should exercise the following precautions when using the kit:

- Use sterile pipette tips with filters.
- Store and extract positive material (specimens, controls and amplicons) separately from all other reagents and add it to the reaction mix in a spatially separated facility.
- Thaw all components thoroughly at room temperature before starting an assay.
- When thawed, mix the components and centrifuge briefly.
- Work quickly on ice.

Quality Control

In accordance with Norgen's ISO 9001 and ISO 13485-certified Quality Management System, each lot of Norgen's *Mycoplasma* PCR Detection Kit, including the Mycoplasma 2x PCR Master Mix, Control 2x PCR Master Mix, Isolation Control and Mycoplasma Positive Control are tested against predetermined specifications to ensure consistent product quality.

Product Use Limitations

Norgen's *Mycoplasma* PCR Detection Kit is designed for research purposes only.

Product Warranty and Satisfaction Guarantee

NORGEN BIOTEK CORPORATION guarantees the performance of all products in the manner described in our product manual. The customer must determine the suitability of the product for its particular use.

Precautions and Disclaimers

This kit is designed for research purposes only. It is not intended for human or diagnostic use.

Safety Information

Ensure that a suitable lab coat, disposable gloves and protective goggles are worn when working with chemicals. For more information, please consult the appropriate Material Safety Data Sheets (MSDSs). These are available as convenient PDF files online at *www.norgenbiotek.com*.

CAUTION: DO NOT add bleach or acidic solutions directly to the sample-preparation waste.

Important Notes Prior to Beginning Protocol:

- A variable speed centrifuge should be used for maximum kit performance. If a variable speed centrifuge is not available a fixed speed centrifuge can be used, however reduced yields may be observed.
- Ensure that all solutions are at room temperature prior to use, and that no precipitates have formed. If necessary, warm the solutions and mix well until the solutions become clear again.
- Prepare a working concentration of the **Wash Solution** by adding 21 mL of 96 - 100 % ethanol (provided by the user) to the supplied bottle containing the concentrated **Wash Solution**. This will give a final volume of 30 mL. The label on the bottle has a box that may be checked to indicate that the ethanol has been added.
- Lysate can be prepared from either cell suspension or monolayer. Please ensure that you follow the proper procedure for lysate preparation in **Step 1a.**
- For the isolation of genomic DNA from
- Preheat a water bath or heating block to 65°C.
- **Isolation Control (*IsoC*)**
    - An Isolation Control (*IsoC*) is supplied. This allows the user to control the DNA isolation procedure. For this assay, add the Isolation Control *(IsoC)* to the lysate during the isolation procedure
    - The Isolation Control *(IsoC)* must not be added to the sample material directly.
    - Do not freeze and thaw the Isolation Control *(IsoC)* more than 2 times.
    - The Isolation Control *(IsoC)* must be kept on ice at all times during the isolation procedure.
- The PCR components of the *Mycoplasma* PCR Detection Kit should remain at -20^{o}C until DNA is extracted and ready for PCR amplification.
- It is important to work quickly during this procedure.

1. Lysate Preparation

a. **From suspension :** Centrifuge 15 - 50 mL of cells growing in suspension for 5 minutes at 3000 x g in order to collect the cell pellet. Decant the supernatant and resuspend the cell pellet with PBS or fresh media to obtain the appropriate number of cells (1 x 10^6 cells).

From monolayer : After trypsinization, collect and wash the cells with PBS or fresh media to obtain the appropriate number of cells (1 x 10^6 cells)

b. Centrifuge at 14,000 x g (~14,000 RPM) for 1 minute to pellet the cells. Pour off the supernatant carefully so as not to disturb or dislodge the cell pellet.

c. Add 500 µL of **Lysis Solution** to the cell pellet. Resuspend the cells by gentle vortexing.

d. Transfer the mixture to a provided **Bead Tube** and secure the tube horizontally on a flat-bed vortex pad with tape, or in any commercially available bead beater equipment (e.g. Scientific Industries' Disruptor Genie™).

e. Vortex for 5 minutes at maximum speed or optimize the condition for any commercially available bead beater equipment.

Note: Foaming during the homogenization is common. This foaming is due to detergents present in the **Lysis Buffer** and will not affect the protocol.

f. Incubate the **Bead Tube** with lysate at 65°C for 10 minutes. Occasionally mix the lysate 2 or 3 times during incubation by inverting the tube.

g. Briefly spin the tube to remove liquid from the cap, and transfer all of the lysate, including cell debris, to a DNase-free microcentrifuge tube (provided by the user) by pipetting. Ensure that the beads are not transferred during the pipetting.

h. Centrifuge the tube for 2 minute at 14000 × g (~14,000 RPM).

i. Carefully transfer clean supernatant to a new DNase-free microcentrifuge tube (provided by the user) without disturbing the pellet. Note the volume.

j. Add an equal volume of 70% ethanol (provided by the user) to the lysate collected above (100 µL of ethanol is added to every 100 µL of lysate). Vortex to mix.

k. Proceed to Step 2: Binding to Column

2. Binding DNA to Column

- **a.** Assemble a spin column with one of the provided collection tubes.
- **b.** Add 10 µL of **Isolation Control (*IsoC*)** to the lysate mixture.
- **c.** Apply up to 600 µL of the lysate with ethanol onto the column and centrifuge for 1 minute at 14,000 x g (~14,000 RPM). Discard the flowthrough and reassemble the spin column with the collection tube.

  Note: Ensure the entire lysate volume has passed through into the collection tube by inspecting the column. If the entire lysate volume has not passed, spin for an additional minute.

- **d**. Depending on your lysate volume, repeat step 2c if necessary.

3. Column Wash

- **a.** Apply 500 µL of Wash Solution to the column and centrifuge for 1 minute.

  Note: Ensure the entire wash solution has passed through into the collection tube by inspecting the column. If the entire wash volume has not passed, spin for an additional minute.

- **b.** Discard the flowthrough and reassemble the column with its collection tube.
- **c.** Repeat step 3a to wash column a second time.
- **d.** Discard the flowthrough and reassemble the spin column with its collection tube.
- **e.** Spin the column for 2 minutes in order to thoroughly dry the resin. Discard the collection tube.

4. DNA Elution

- **a.** Place the column into a fresh 1.7 mL Elution tube provided with the kit.
- **b.** Add 75 µL of Elution Buffer to the column.
- **c.** Centrifuge for 2 minutes at 2,000 x g (~2,000 RPM), followed by a 1 minute spin at 14,000 x g (~14,000 RPM). Note the volume eluted from the column. If the entire volume has not been eluted, spin the column at 14,000 x g (~14,000 RPM) for 1 additional minute.

5. Storage of DNA

The purified nucleic acids may be stored at –20°C for a few days. It is recommended that samples be placed at –70°C for long term storage.

B. Mycoplasma PCR Assay Preparation

Notes:

- Before use, suitable amounts of all PCR components should be completely thawed at room temperature, vortexed and centrifuged briefly.
- The amount of Mycoplasma 2X Detection PCR Master Mix and Control 2X PCR Master Mix provided is enough for up to 32 PCR reactions (24 sample PCR, 4 positive control PCR and 4 no template control PCR).
- For each sample, one PCR reaction using the Mycoplasma 2X Detection PCR Mastermix and one PCR reaction using Control 2X PCR Mastermix should be set up in order to have a proper interpretation of the results.
- For every PCR run, one reaction containing Mycoplasma Positive Control and one reaction as no template control must be included for proper interpretation of results.
- The recommended minimum number of DNA samples tested per PCR run is 6.
- Using a lower volume from the sample than recommended may affect the sensitivity of Mycoplasma Limit of Detection.

1. Prepare the PCR reaction for sample detection (Set #1, using **Mycoplasma 2X Detection PCR Mastermix**) and the PCR reaction for control detection (Set #2, using **Control 2X PCR Mastermix**) as shown in Table 1 below. The recommended amount of sample DNA to be used is 2.5 µL.

However, a volume between 1 and 5 µL of sample DNA may be used as template. Ensure that one Mycoplasma detection reaction and one control reaction is prepared for each DNA sample. Adjust the final volume of the PCR reaction to 20 µL using the Nuclease-Free Water provided.

Table 1. PCR Assay Preparation

PCR Components	Volume Per PCR Reaction
Mycoplasma 2X PCR Master Mix Or Control 2X PCR Master Mix	**10 µL**
Sample DNA	**2.5 µL**
Nuclease-Free Water	**7.5 µL**
Total Volume	**20 µL**

2. For each PCR set, prepare **one** positive control PCR as shown in Table 2 below:

Table 2. PCR Positive Control Preparation

PCR Components	Volume Per PCR Reaction
Mycoplasma 2X PCR Master Mix Or Control 2X PCR Master Mix	**10 µL**
Mycoplasma *Positive Control (PosC)*	**10 µL**
Total Volume	**20 µL**

3. For each PCR set, prepare **one** no template control PCR as shown in Table 3 below:

Table 3. PCR Negative Control Preparation

PCR Components	Volume Per PCR Reaction
Mycoplasma 2X PCR Master Mix Or Control 2X PCR Master Mix	**10 µL**
Nuclease-Free Water	**10 µL**
Total Volume	**20 µL**

C. Mycoplasma PCR Assay Programming

1. Program the thermocylcer according to the program shown in Table 4 below.
2. Run one step PCR.

Table 4. *Mycoplasma* Assay Program

PCR Cycle	Step	Temperature	Duration
Cycle 1	Step 1	95°C	3 min
Cycle 2 (40x)	Step 1	94°C	15 sec
	Step 2	60°C	15 sec
	Step 3	72°C	30 sec
Cycle 3	Step 1	72°C	5 min
Cycle 4	Step 1	4°C	∞

D. *Mycoplasma* PCR Assay Results Interpretation

1. For the analysis of the PCR data, the entire 15-20 µL PCR Reaction should be loaded on a 1X TAE 1.7% Agarose DNA gel along with 10 µL of Norgen's DNA Marker (provided).
2. The PCR products should be resolved on the 1X TAE 1.5% Agarose gel at 150V for 30 minutes (Gel running time will be vary depending on an electrophoresis apparatus).
3. Sample results are provided below:

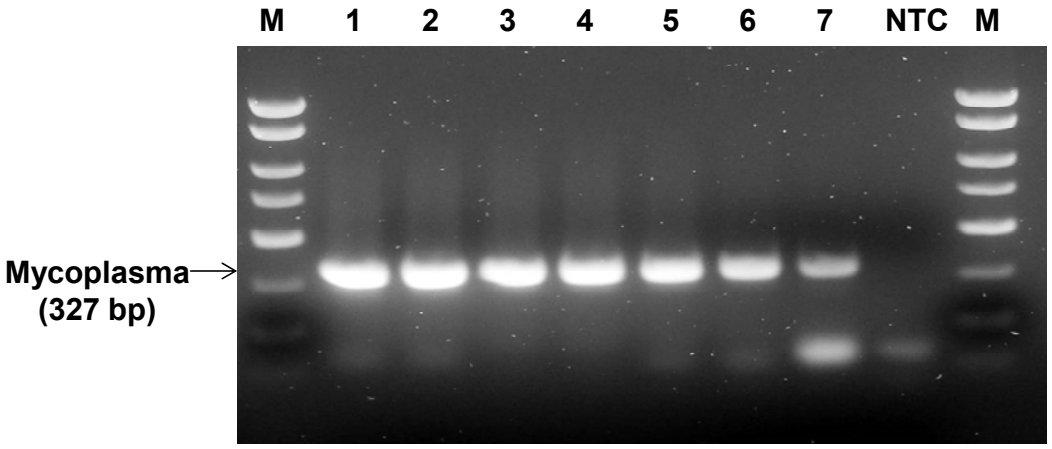

Figure 1: A representative 1X TAE 1.5% agarose gel showing the amplification of *Mycoplasma* at different concentrations (Lane 1 to 7). The size of the *Mycoplasma* target amplicon corresponds to 327 bp as represented by the provided DNA Marker (M). **NTC** = Negative Control.

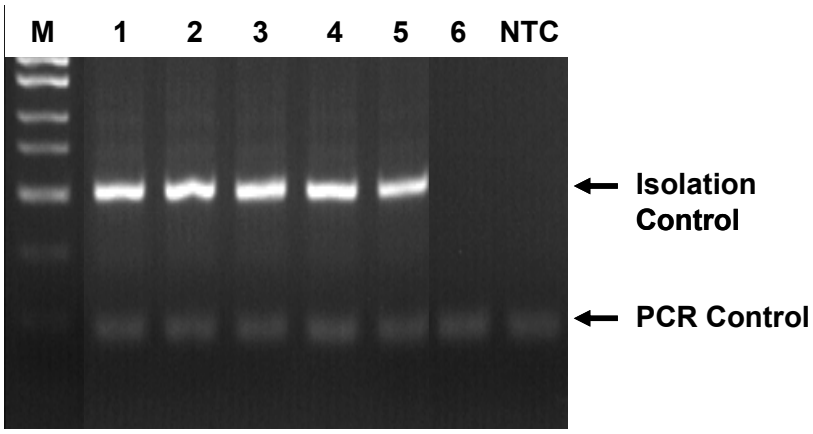

Figure 2: A representative 1X TAE 1.7% agarose gel showing the amplification of **Isolation Control** and **PCR Control** under different conditions using the **Control 2X PCR Mastermix**. The size of the Isolation Control amplicon and PCR Control amplicon correspond to 499 bp and 150 bp, respectively, as represented by the provided DNA Marker (M). Lanes 1 to 5 showed detection of both Isolation Control and PCR Control, suggesting that the DNA isolation as well as the PCR reaction was successful. Lane 6 showed only the detection of PCR Control suggesting that while the PCR was successful, the isolation failed to recover even the spiked-in Isolation control. **NTC** = Negative Control.

Table 5. Interpretation of PCR Assay Results

Input Type	**Target reaction**	**Control Reaction**		**Interpretation**
	Mycoplasma Target Band (327 bp)	***IsoC* Band (499 bp)**	***PCRC* Band (150 bp)**	
Positive Control	X	X	X	Valid
Negative Control			X	Valid
Sample	X	X	X	Positive
Sample		X	X	Negative
Sample			X	Re-test
Sample				Re-test
Sample		X		Negative
Sample	X		X	Positive
Sample	X	X		Positive
Sample	X			Re-test

****** For results obtained that are not covered in Table 5 above, please refer to the Troubleshooting Section.

E. *Mycoplasma* PCR Assay Specificity and Sensitivity

- The specificity of Norgen's *Mycoplasma* PCR Detection Kit is first and foremost ensured by the selection of the *Mycoplasma* specific primers, as well as the selection of stringent reaction conditions. The primers were checked for possible homologies to all in GenBank published sequences by sequence comparison analysis. The specific delectability of all relevant strains has thus been ensured by a database alignment and by PCR amplification with the following bacteria commonly found in filed samples.
    - *Listeria*
    - *Salmonella*
    - *Streptococcus agalactiae*
    - *Streptococcus dysglactiae*
    - *Streptococcus uberis*
    - *Staphylococcus aeurus*
    - *E.coli*
    - *Mycoplasma*

F. Linear Range

- The linear range (analytical measurement) of Norgen's *Mycoplasma* PCR Detection Kit was determined by analysing a dilution series of a *Mycoplasma* quantification standards ranging from 1 x 10^6 cfu/µl to 1 x 10^1 cfu/µl.
- Each dilution has been tested in replicates (n = 4) using Norgen's *Mycoplasma* PCR Detection Kit on a 1X TAE 1.7% agarose gel.
- The linear range of Norgen's *Mycoplasma* PCR Detection Kit has been determined to cover concentrations from 24 fg to 2.4 ng
- Under the conditions of the Norgen's *Mycoplasma* DNA Isolation procedure, Norgen's *Mycoplasma* PCR Detection Kit covers a linear range from 24 copies to 2.4 x 10^5 copies.

Frequently Asked Questions

1. How many samples should be included per PCR run?

- Norgen's *Mycoplasma* PCR Detection Kit is designed to test 24 samples. For every 6 samples, a non-template control (Nuclease Free Water) and a Positive Control must be included. It is preferable to pool and test 6 samples at a time. If not, the provided Positive Control is enough to run 3 samples at a time.

2. How can I interpret my results if neither the PCR control nor the Isolation Control (*IsoC*) amplifies?

- If neither the PCR control nor the Isolation Control (*IsoC*) amplifies, the sample must be re-tested. If the positive control showed amplification, then the problem occurred during the isolation, where as if the Positive control did not amplify, therefore the problem has occurred during the setup of the PCR assay reaction.

3. How should it be interpreted if only the PCR control showed amplification but neither the *Mycoplasma* target nor the Isolation control amplified for a sample?

- This indicates a poor isolation. The isolation procedure must be repeated.

4. How should it be interpreted if only the Isolation Control (*IsoC*) was amplified in a sample?

- The sample tested can be considered as *Mycoplasma* negative.

5. How should it be interpreted if the PCR control and the *Mycoplasma* target showed amplification in a sample?

- The sample tested can be considered positive. It could happen when too much template was added to the reaction.

6. How should it be interpreted if only the *Mycoplasma* target and the PCR control were amplified in a sample?

- The sample tested can be considered as *Mycoplasma* positive.

7. How should it be interpreted if only the *Mycoplasma* target was amplified in a sample?

- It is recommended that the isolation is repeated.

8. How should it be interpreted if only the PCR control and the Isolation control showed amplification in a sample?

- The sample tested can be considered negative

9. What if I forgot to do a dry spin after my third wash?

- Your first DNA elution will be contaminated with the Wash Solution. This may dilute the DNA yield in your first elution and it may interfere with the PCR detection, as ethanol is known to be a PCR inhibitor.

10. What if I forgot to add the Isolation Control (*IsoC*) during the isolation?

- It is recommended that the isolation is repeated.

11. What if I forgot to run the Control PCR for the sample and I only ran the Detection PCR and I obtained a positive result?

- The result can be considered positive. However, any negative result must be verified by running the associated control PCR to ensure that it is a true negative and not a false negative due to problems with the DNA isolation or the PCR reactions.

Related Products	Product #
Fungi/Yeast Genomic DNA Isolation Kit	27300
Bacterial Genomic DNA Isolation Kit	17900
Plant/Fungi DNA Isolation Kit	26200

Technical Assistance

NORGEN's Technical Service Department is staffed by experienced scientists with extensive practical and theoretical expertise in sample and assay technologies and the use of NORGEN products. If you have any questions or experience any difficulties regarding Norgen's *Mycoplasma* PCR Detection Kit or NORGEN products in general, please do not hesitate to contact us.

NORGEN customers are a valuable source of information regarding advanced or specialized uses of our products. This information is helpful to other scientists as well as to the researchers at NORGEN. We therefore encourage you to contact us if you have any suggestions about product performance or new applications and techniques.

For technical assistance and more information, please contact our Technical Support Team between the hours of 8:30 and 5:30 (Eastern Standard Time) at (905) 227-8848 or Toll Free at 1-866-667-4362 or call one of the NORGEN local distributors (www.norgenbiotek.com) or through email at techsupport@norgenbiotek.com.

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